

# Mast Cells Shape Early Life Programming of Social Behavior

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## Undergraduate Research Thesis

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Inflammation during development increases risk for neuropsychiatric disorders, including autism. Mast cells are a type of innate immune cells present in the brain that modulate inflammation. However, their role in brain development is unknown. We have previously shown thousands of mast cells reside in the developing rat brain suggesting that they may mediate the effects of early life inflammation. The goals of these experiments were to characterize effects mast cells play on the development of social behaviors. To determine if mast cells participate in regular programming of social behaviors, we pharmacologically activated or inhibited mast cells during the neonatal period and tested social interaction behavior in adulthood. We found that social avoidance was decreased in males following mast cell activation. There were no effects on active investigation or passive interaction. Early life activation led to life-long increases in the brain mast cell population. We next utilized a prenatal allergic immune challenge to better simulate physiologically-relevant mast cell activation during development. We performed a social play test on juvenile rats, and found that they engaged in less social play behavior than the control group. We are currently testing the effects of allergic challenge on social interaction behavior in adult rats. We are also assessed the effects on allergic challenge on the number of mast cells in the in brain regions, and found that hippocampal mast cell up-regulation with allergic challenge correlates to the observed behavioral decreases in male juvenile play, however females also showed a decrease in rough and tumble behaviors that did not correlate with mast cell differences. Because neuropsychiatric disorders are more prevalent in males and our lab has previously found marked sex differences in the number of mast cells in the developing brain, we determined that males and females respond differently to prenatal allergic challenge. Together these studies show that mast cells may play a key role in the early life programming of social behavior, and activation of these cells following early life inflammation may contribute to the ontogeny of autism.

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## **Introduction**

### **Early life inflammation is a risk factor for neurodevelopmental disorders**

According to the World Health Organization, over 25% of the global population will develop some form of mental or neurological disorder in their lifetime (World Health Organization, 2001). Neuropsychiatric disorders such as autism spectrum disorders (ASDs) and schizophrenia remain serious conditions affecting many families in the United States today. Perinatal inflammation and biological sex are two risk factors that contribute to the onset of these disorders (Newschaffer et al., 2007). In human mothers, infection, regardless of bacterial or viral origin, during the time of pregnancy increase the risk of ASD two fold (Atladottir et al., 2012). Furthermore, perinatal inflammation is also common in preterm infants, who also display an increased risk for ASDs (Dammann and Leviton, 2000; Johnson et al., 2010). This inflammation typically presents itself throughout life both in the periphery and the central nervous system (CNS) of ASD patients (Vargas et al., 2005; Ashwood et al., 2011). The higher prevalence of allergies and autoimmune disorders seen in autistic individuals also supports immune dysfunction as an underlying risk factor (Careaga et al., 2010). Males present ASDs approximately four times more often than females, suggesting that males are highly sensitive to developmental disturbances (Werling and Geschwind, 2013). Unfortunately, no known cure exists and the efficacy of treatments varies greatly depending on the individual (Myers and Johnson, 2007). While genetics play a critical role in the development of many neuropsychiatric disorders (Colvert et al., 2014), the underlying physiological processes of such disorders remain unknown (Werling and Geschwind, 2013). Without knowing the cause of the disease, there is little hope of finding a cure.

Rodents subjected to early life inflammation exhibit behavioral similarities to the autistic phenotype (Silverman et al., 2010). Several types of cells regulate this immune response in the brain. Historically, the astrocytes and microglia were thought of as the main contenders for neuronal inflammation, but recent evidence suggests a third type of cell, known as the mast cell, plays a critical role in regulating neuroinflammatory processes as well (Silver and Curley 2013). However, no research has looked at how mast cells influence neuronal development or how they impact later life behavioral changes. For the first time, we look at how perinatal inflammation influences mast cell populations in the brain regions associated with social interaction, and how these differences may contribute to life-long behaviors in rats.

Compelling evidence from both clinical and laboratory studies show a strong connection in several psychiatric diseases that link the immune system with disorders that also exhibit a developmental origin (Schwarz and Bilbo, 2012a). Perinatal immune activation, during a time when immune cells are especially plastic, alter long-term function of the immune cells and lead to negative changes in memory and learning in males (Schwarz and Bilbo, 2012b). These findings reveal that environmental factors impact the immune system and its role in cognitive development. While glial cells have been linked to Alzheimer's disease, Parkinson's disease, schizophrenia and autism, almost nothing is known of the developmental role that mast cells play in the ontogeny of these disorders (Silver and Curley, 2013; Patel et al., 2013).

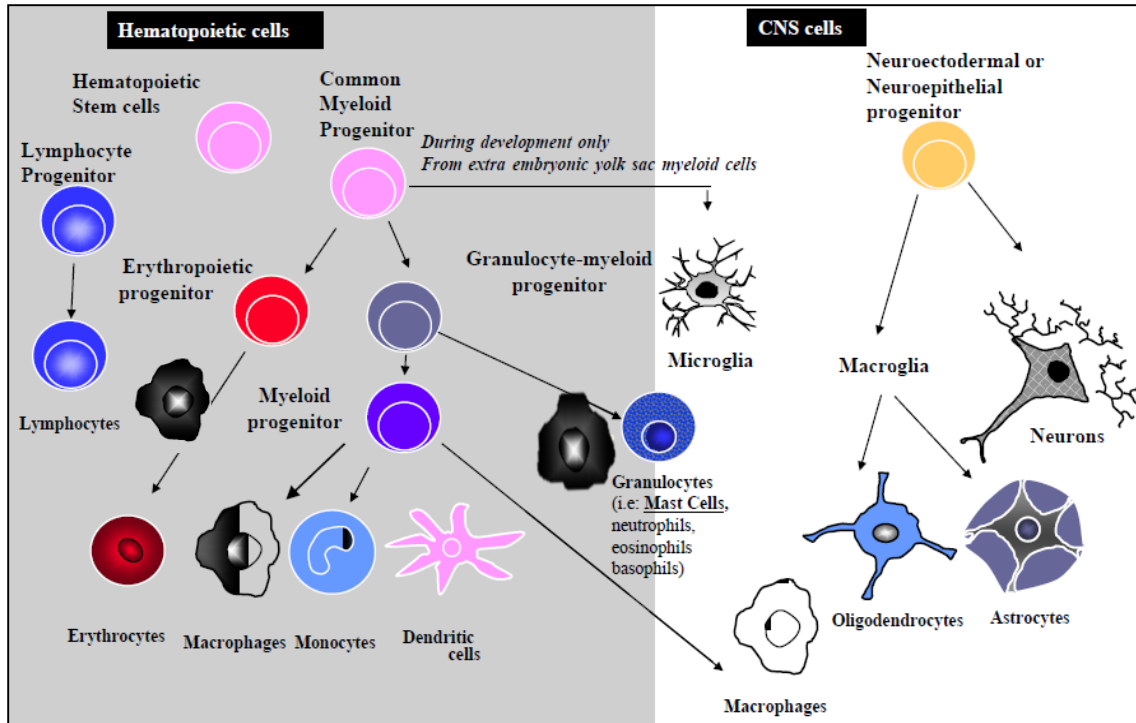
### **Function of the CNS immune system during development**

The innate immune system is defined as the nonspecific immune system. In the periphery, specialized cells of this system include natural killer cells, neutrophils, monocytes, dendritic cells, macrophages and mast cells, which act as the first line of defense against invading pathogens. These cells can identify foreign proteins and either engulf and destroy them

via a process called phagocytosis or use chemical mediating-signals known as cytokines to recruit other immune cells (Grasso, 2012). The CNS also possesses innate immunity, but utilizes specialized cells for purposes beyond the typical pathogen recognition.

In the adult brain, the parenchyma is regarded as an immune privileged site, meaning that inflammatory cells typically found in the periphery cannot freely enter or exit to fight infection. This reduced immune trafficking combined with the immunosuppressive environment of the brain serves as a neuroprotective mechanism (Bechmann and Perry, 2007). Such an environment is necessary because excess neuroinflammation can damage the neurons and lead to cognitive and motor deficits (Robert, 2007). As a result, a specialized subset of immune cells resides in the CNS, which includes microglia, astrocytes, and mast cells. These cells modulate neuroinflammation through the release of cytokines, chemokines, and oxidative stress. (Silver and Curley, 2013)

Microglia are the primary innate immune cell of the CNS. These non-neuronal cells are derived from common myeloid progenitor cells of the yolk sac which infiltrate the brain around gestational day 8 (GD8) in rats (Alliot et al., 1999; Ginhoux et al., 2010) The myeloid progenitors then differentiate into microglia (**Figure 1**), proliferate during the perinatal period, and remain a relatively stable population throughout life that is distinct from other peripheral macrophages (Alliot et al., 1999; Ginhoux et al., 2010). While historically being associated with debris collection and typical pathogen resistance in the brain, microglia also play a unique and critical role in brain formation and function (Lenz and McCarthy, 2014).



**Figure 1:** Origin of innate immune cells (Adapted from lecture by Godbout, 2014). Mast cells are a myeloid derived granulocyte of the innate immune system. They are present in both the periphery and brain of rats, mice, humans, and other mammalian species.

Microglia also play an important role in development through cell proliferation, survival, apoptosis, and synaptic pruning (Battista et al., 2006). In vitro studies showed that microglia are essential to induce neurogenesis in subventricular neuroprogenitor cells (Walton et al., 2006). Furthermore, by pharmacologically suppressing the proinflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ , INF- $\gamma$ ) released by microglia in development, neurogenesis was reduced in the area, suggesting that microglia-derived proinflammatory cytokines enhance neurogenesis (Shigemoto-Mogami et al., 2014). On the other hand, in highly proliferative zones, microglia prevent neuronal overpopulation through phagocytosing both apoptotic as well as healthy neural precursor cells (Cunningham et al., 2013). Microglia are also drawn to the apoptotic cells of the developing brain and release reactive oxygen species to complete the cell death process



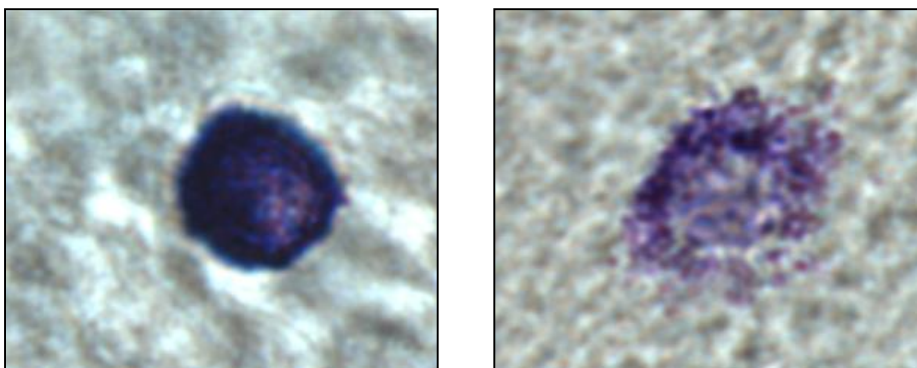
(Wakselman et al., 2008). Marin-Teva and others showed a similar process in the hippocampus and cerebellum, where microglia were attracted to the cell death chemical signal of caspase-3 (Marin-Teva et al., 2004). This cellular pruning ability also helps develop synapses in the hippocampus. Paolicelli detected microglia phagocytosing post-synaptic dendritic spines, which relies on the microglia-neuron fractalkine signaling (Paolicelli et al., 2011). Deficits in synaptic pruning, a critical process for development, lead to abnormal or weak neuronal connectivity and are implicated in the behavioral changes associated with autism (Zhan et al., 2014; Courchesne et al., 2005). Using a rodent fractalkine knockout model (Cx3cr1<sup>KO</sup>) which prevents microglial elimination of immature synaptic connections during development, Zhan and others demonstrated that a primary deficit in pruning is sufficient to induce long term behavioral characteristics similar to autism, such as impaired social interaction and repetitive behaviors (Zhan et al., 2014). Additionally, brain regions typically associated sex specific behaviors, such as the POA and hippocampus, have been shown to have a greater population of microglia in males than in females, demonstrating one possible mechanism for sexually dimorphic behaviors (Courchesne et al., 2005; Lenz, Nugent, et al., 2013; Lenz, Pickett, et al., 2013). However, microglia are not the only type of immune cell in the brain. The mast cell is a bone marrow derived immune cell that is prevalent in the developing brain and releases neuroactive compounds, but its action during development has gone largely uncharacterized (Silver and Curley, 2013).

## **Mast Cells**

A mast cell is another myeloid derived effector cell of the innate immune system that promotes the initial response to injury or infection (Skaper et al., 2012). Historically, mast cells have been associated with atopic allergy, asthma, and anaphylaxis. In the periphery, they reside

close to epithelia, blood vessels, nerves, smooth muscle cells, in the airways, and in the gastrointestinal tract (Metcalf et al., 1997). Following injury, they can release proinflammatory molecules, or, depending on the environment, act to modulate inflammation (Nelissen et al., 2013). Depending on the location, the phenotype of the mast cells can vary, resulting in differing functional properties (i.e. inflammatory, immunological, or physiological) (Bienenstock et al., 1985; Kitamura, 1989; Galli, 1990). Moreover, mast cells have been observed in the human brain and are known to produce histamine and serotonin (Church and Levi-Schaffer, 1997).

Activation of the mast cells is modulated by cytokines, growth factors, and other microenvironmental signals (Galli, 1990). One common method of activation and the type described in our study utilizes IgE (type E immunoglobulin) and its activation of the FcεRI receptor (Stone et al., 2010), causing the release of histamine, serotonin, cytokines, or enzymes through a process known as degranulation (**Figure 2**). In addition to IgE, mast cell number and activation can be influenced by hormones like corticotrophin-releasing hormones (CRH) and gonadal steroids, neuropeptides and nerve growth factors (NGF) (Crompton et al., 2003; Kulka et al., 2008; Lambracht-Hall, 1990).



**Figure 2:** Mast cell before releasing regulatory molecules (Left) and after releasing molecules (Right). Mast cells are associated with allergy and asthma responses in the brain, releasing serotonin, enzymes, and other critical regulatory molecules. (Lenz).

Mast cells release many neuroactive substances. Degranulation causes the release of preformed mediators located in cytoplasmic granules, including vasoactive amines, neutral proteases, proteoglycans, cytokines, and growth factors (Dvorak, 1992; Dvorak et al., 1994). Mast cells also synthesize proinflammatory lipid mediators, such as prostaglandins and leukotrienes, *de novo* (Gilfillan et al., 2011). Finally, mast cells synthesize and secrete growth factors, cytokines, and chemokines in response to various stimuli (Gilfillan et al., 2011) (**Table 1**).

**Table 1:** Summary of many known compounds mast cells are capable of releasing upon activation.

(Adapted by Galli et al., 2005 from Weller et al., 2003)

Major mediators stored in preformed in cytoplasmic granules	Histamine, serotonin (in murine rodents), heparin and/or chondroitin sulfates, neutral proteases (chymases and/or tryptases), major basic protein, many acid hydrolases, cathepsin, carboxypeptidases, peroxidase
Major lipid mediators produced on appropriate activation	PGE2, PGD2, LTB4 and LTC4, platelet-activating factor
Cytokines released on appropriate activation	TNF <sup>a</sup> , TGF- $\beta$ , MIP-1 $\alpha$ , VPF/VEGF <sup>a</sup> , FGF-2 <sup>a</sup> , LIF, IFN- $\alpha$ , IFN- $\beta$ , IFN- $\gamma$ , GMCSF, MCP-1, IL-1 $\alpha$ , IL-1 $\beta$ , IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-9, IL-11, IL-12, IL-13, IL-15, IL-16, IL-18, IL-25 (and probably many more)
Anti-microbial peptides	Cathelicidin (human mast cells: LL-37; murine mast cells: CRAMP <sup>b</sup> )

<sup>a</sup> Evidence indicates that certain cytokines produced by mast cells, such as tumor necrosis factor (TNF), fibroblast growth factor (FGF)-2 and vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF), are released in part from preformed stores, some of which may be physically associated with the cells' cytoplasmic granules.

<sup>b</sup> CRAMP: cathelin-related antimicrobial peptide.

Since they can release biologically active mediators in response to IgE, mast cells are typically thought of as the primary effector cells underlying allergic disorders (Gilfillan et al.,

2011). The IgE-induced degranulation can happen within minutes (Galli, 1990). As a result, mast cells play a vital role in mediating the immediate effects of anaphylactic and atopic reactions. Though evidence for mast cell linkage to chronic conditions like asthma is not completely understood (Williams and Galli, 2000), the mast cell stabilization drug, sodium cromolyn, has been used for many years to treat asthma (Murphy and Kelly, 1987).

Aside from their role in the periphery, recent studies have begun to focus on their role in the brain. Just as the microglia progenitor cells migrate to the brain, mast cells take up residence in the developing brain as well (Lambrecht-Hall et al., 1990). Mast cells can also pass through the blood-brain barrier (BBB) under normal conditions and lie on the brain side near the blood vessels (Silverman et al., 2000). They sense the local environment and will release different neuroactive compounds (Galli et al., 2005), including histamine, prostaglandins, growth factors,  $\text{TNF-}\alpha$ , IL-6, IL-13, and serotonin even during baseline conditions (Skaper et al., 2012; Kim et al., 2010, Walker et al., 2012). In the developing rat brain, the cells primarily populate the velum interpositum, the area directly underneath the hippocampus, and peak in number at postnatal day 4 (PN4) (Tuomisto and Panula, 1991).

While brain-resident mast cell counts are relatively small compared to the numbers of brain-resident microglia and astrocytes, they release a large amount of preformed granular material (picograms per cell) or continue synthesizing mediators for several hours following activation, allowing for prolonged effects on the glial and neuronal cells (Silver and Curley, 2013). For example, activated mast cells can produce proinflammatory chemokines, which induce an activated profile in microglia (Marshall, 2004). The microglia then release factors like Interleukin 6 (IL-6) and CCL5 which could alter the receptor expression on the mast cells

(Pietrzak et al., 2011). There is likely a high degree of crosstalk between mast cells and other immunocompetent cells in the brain that have yet to be characterized.

Alterations of mast cell number and activation state can lead to behavioral changes. In mast cell deficient genetic model mice, neurogenesis and hippocampal dependent behaviors in adults were negatively affected, but treatment with a serotonin selective reuptake inhibitor reversed the effects (Nautiyal et al., 2012). The findings support the idea that mast cells contribute to proper function of the hippocampus through serotonergic release even in the absence of inflammation. However, behavioral changes linked to differences in mast cell number and activation likely results from a change in the balance of the many mediators present in the brain so a simple linear relation between number, degranulation, and behavioral differences is improbable (Silver and Curley, 2013). No studies have looked at the impact of acute mast cell manipulations during development, or assessed whether behavioral changes seen in congenitally mast cell deficient mice are due to the developmental or adult actions of brain-resident mast cells.

### **Serotonin, histamine, and other mast cell mediators are implicated in brain development and function**

Many of the compounds mast cells produce have been shown to be essential for proper brain development. Serotonin (5-HT), for instance, is one of the most widely studied neurotransmitters and an influential tropic factor during development of the mammalian brain. In a study using genetic knockout mice, serotonin was determined to be a necessary factor for properly shaping embryonic neuronal structures (Cote et al., 2007). However, excess embryonic serotonin also leads to abnormal cortical development and neuronal migration. In the postnatal brain, disruptions of synaptic connectivity result from altered serotonin levels prior to puberty (Ricco et al., 2009; Chugani et al., 1999). Experiments in rat pups determined that

pharmacological depletion of serotonin lead to a lengthened period of cell division in brain regions with dense serotonergic innervations, resulting in an increase of neuronal cell numbers in the hippocampus, superior colliculus, and several thalamic nuclei (Lauder and Krebs, 1978). Recent findings in a rodent model concluded that serotonin requires a homeostatic balance during development to provide a necessary signal for serotonergic neuronal wiring, and abnormalities in this balance negatively affect this neuronal circuitry in areas like the hippocampus and thalamic nuclei (Migliarini et al., 2013). These results reflect observations in human children. All children typically have elevated serotonin levels until 15 years of age, but autistic patients often experience difficulties in their capacity to synthesize this neurotransmitter (Chugani et al., 1999). Improper development of areas shaped by serotonin leads to adverse behavioral characteristics resembling autism (Whitaker-Azmitia et al., 2005). When treated with a serotonin transport blocker to increase levels, rats exposed in early life experienced a change in exploratory and stress response behaviors while adults that underwent the same treatment did not experience any change, implying that the effects are exerted during a developing stage of life (Ansorge et al., 2008).

Histamine (HA) in the adult brain regulates behaviors including the sleep-wake cycle, feeding, and neuroendocrine function. In development, histamine levels typically are elevated compared to the adult brain, suggesting a role in proper brain formation as well (Pearce and Schanberg, 1969). Most histamine in the developing brain has been attributed to mast cells (Tuomisto and Panula, 1991) and genetically blocking mast cell development reduces brain histamine levels (Nautiyal and others, 2008). The mast cells that contain histamine are first observed on gestational day 18-19 and mainly reside directly underneath the developing hippocampus (Tuomisto and Panula, 1991). Cell numbers increase until PN 4, after which their

number declines towards the adult level (Tuomisto and Panula, 1991). Histamine deficits have been implicated in the developmental disorder Tourette's syndrome, and recent findings showed that a genetic knockout developed to block histamine synthesis through altered enzyme function lead to a phenotype of the disease in a mouse model including frequent repetitive motions, also a characteristic of autistic behavior (Castellan et al., 2014). The same study also found that humans with the correlating genetic polymorphism were at a high risk for developing the disorder.

In vitro studies looking at the neural stem cells observed that histamine acts to induce proliferation and neuronal differentiation of neural stem cells (Molina-Hernandez, 2008). While neural stem cells are known to express histaminergic receptors, receptor knockout mice do not exhibit developmental abnormalities from lack of histamine (Molina-Hernandez, 2008). Overall, however, the role of histamine in the developing CNS is not yet well characterized (Panula and Nuutinen, 2013).

### **Sex differences in brain-resident innate immune cells**

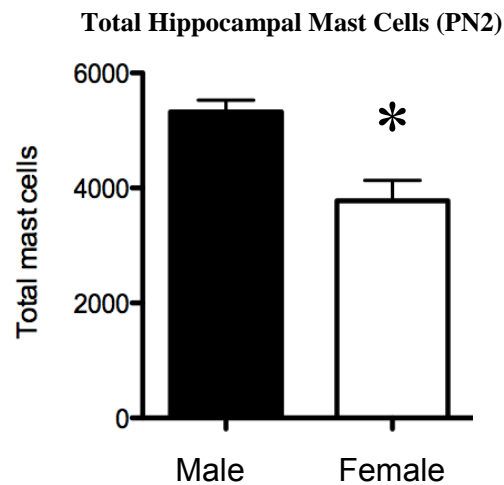
Mast cells have been shown to play a significant role in development, central nervous system immunological inflammatory responses, and everyday physiological functioning through the release of neuromodulatory chemicals (Silver and Curley, 2013). After fertilization, hormones direct mast cell activity through differentiation in the developing fetus that leads to the development of characteristics common to each gender. Previous and ongoing work from our laboratory shows that if mast cell activation is increased during this early organization phase of brain sexual differentiation, male-typical copulatory behavior will be observed in female rats, months later in adulthood (Lenz and McCarthy, 2014). Males also exhibit a much greater number of both microglia and mast cells than the females (**Figure 3**), with the males showing lifelong differences in microglia (Lenz, Nugent, et al., 2013).

With these changes in mind, it is interesting to note that many neuropsychiatric disorders, in particular those with developmental origin, present themselves much more frequently in males. Autism, schizophrenia, ADHD, and Tourette's syndrome all develop more frequently in males and seem to have some inflammatory component underlying their onset. Males typically present

developmental abnormalities early in life in conditions like autism or schizophrenia, while females tend to present symptoms later in life like depression and anxiety disorders (Schwarz and Bilbo, 2012). We hypothesize this difference may play a role in the sex bias of ASDs.

### **Disrupted social behavior is a hallmark phenotype for neuropsychiatric disorders**

While inflammation in the brain has been shown as a risk factor for developing neuropsychiatric disorders in humans, behavioral changes remain the current reliable diagnostic criteria (Silverman et al., 2010). According to the DSM V, three main diagnostic criteria of autism include deficits in social interaction and communication, restricted or repetitive behaviors (Silverman et al., 2010; American Psychiatric Association, 2013) and exhibit symptoms during early development. Abnormal social interaction consists of behaviors like reduced interest in peers, difficulty maintaining social interactions, and failure to use appropriate eye contact or facial expressions. Impaired communication relates to difficulties in verbal communication ranging from comprehension of words or understanding of verbal context to exaggerated or



**Figure 3:** Significant sex differences in the number of hippocampal mast cells on PN2



monotonous speech. Repetitive or restricted behaviors include rituals, compulsions, routine preservation, and narrowed interests (Silverman et al., 2010). However, no single biological marker has been identified (Voineagu et al., 2011); hence, behavioral assessment remains the widespread diagnostic tool (Johnson and Myers, 2011). In our study, we use the rat, a highly social species, as a model to assess the behavioral changes that result following pharmacological or allergic activation of mast cells, with a focus on the social behavior aspect of ASDs.

Our study chose to focus on the social behavior changes in rats that correlate to the diagnostic criteria. To do so, we utilized a rodent model and looked at the standard parameters used to assess behavior (Silverman et al., 2010). Behavioral assays primarily focus on reciprocal social interaction. We did not assess their inclination for repetitive behaviors or impaired communication. Our lab is assessing behaviors that have been shown to be impaired in the currently accepted transgenic rodent models of autism (Silverman et al., 2010; Zhan et al., 2014). LPS-induced perinatal inflammation altered the behavior in a similar manner (Taylor et al., 2012). Our model aims to illustrate the autistic behavioral phenotype by examining the environmental factors thought to play a risk factor in modulating social behavior.

### **Social play shows a robust sex difference**

Social play is the type of social behavior that develops in juvenile rats around PN18 and continues throughout puberty (Meaney, 1981). These behaviors are thought to prepare the rats for adaptive behaviors in adulthood (Auger and Olesen, 2009). In both humans and rodents, males are predisposed to more frequent rough and tumble play behaviors (Auger and Olesen, 2009; Jarvis, 2007). During development, different hormone levels in males and females lead to differing number of glial cells in the amygdala which lead to masculinized play behaviors in female rats, though male behavior was unaffected (Krebs-Kraft et al., 2010). Lesions to the

neonatal amygdala drastically decreased male play behavior, reducing them to the same level as the female control animals (Meaney et al., 1981).

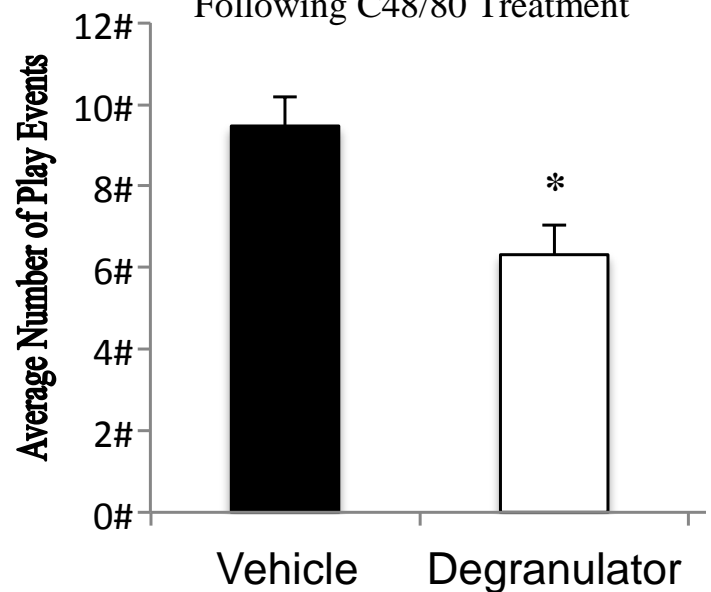
Several other areas of the brain are thought to be associated with modulating social behavior, including the bed nucleus of the stria terminalis (BNST; a part of the extended amygdala), the ventral hippocampus, habenula, and septum. Since the BNST shares strong connections with the amygdala, lesions here would likely alter behavior, though little work has been done to show this (Auger and Olesen, 2009). Male rats treated with LPS prenatally had observable differences in vasopressin expression in the amygdala as well as reduced social play behaviors while females did not show the same differences, demonstrating both the role of the BNST in behavior and the male vulnerability to inflammation (Taylor et al. 2012). Using optogenetics, one study demonstrated that inhibiting projections from the basolateral amygdala to the central amygdala induced anxiety behavior in rats with the open field and elevated plus maze tests (Tye et al., 2011). Alternatively, lesions to the neonatal septum areas increased the social play across both genders though males retained higher play bouts (Beatty et al., 1982). The amygdala also projects to the ventral hippocampus. Previous work has shown excitatory inputs from the basolateral amygdala to the ventral hippocampus down-regulate social behavior (Feliz-Ortiz, 2014). Furthermore, the ventral hippocampus plays a role in anxiety behaviors. Specifically, lesions to the ventral hippocampus, and not the dorsal hippocampus or amygdala, reduce anxiety behavior (McHugh, 2004). This suggests involvement of a pathway affiliated with the decreased social interaction seen in ASD patients. Recent findings suggest that deficits in the CA2 region of the hippocampus impair social learning (DeVito et al., 2009). Many mast cells reside under the CA2 area of the hippocampus during development, which implies a possible interaction.

Van Kerkhof demonstrated that the habenula processes a reward signal affiliated with social play, particularly in young rats (Van Kerkhof, 2013). Pharmacological lesions in the habenula produced social isolation, suggesting this region's possible role in the social impairments of autistic behaviors (Van Kerkhof, 2013).

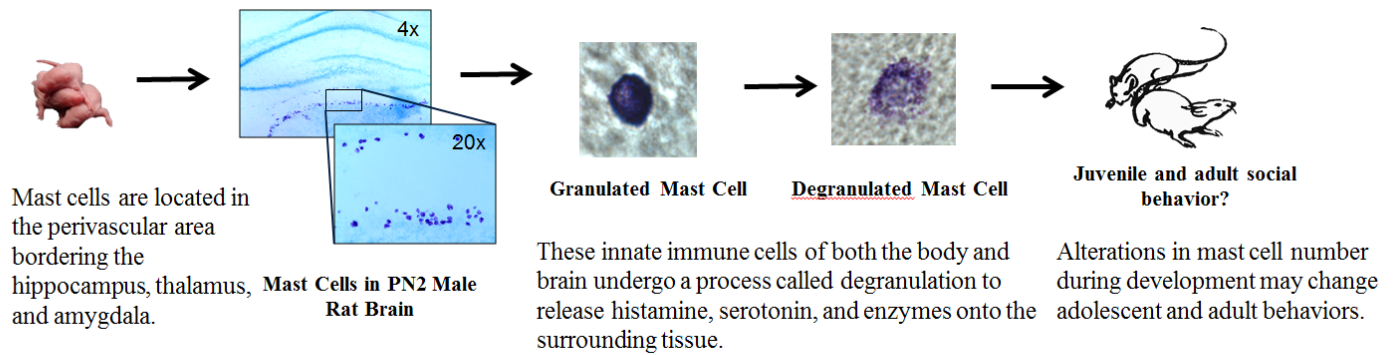
These series of experiments aimed to characterize a role mast cells play during development and their influence on shaping the brain to program the development of social behavior. Previously, our lab looked at juvenile social play behavior after treating pups with compound 48/80, the same mast cell activating drug used in this study. We found a 40% reduction in male social play (Lenz and Galen, unpublished; **Figure 4**).

We wanted to expand upon this work utilizing pharmacological manipulations to directly observe mast cell influences on later life behavior. The following test used a more biologically relevant prenatal immune activation before observing behaviors (**Figure 5**). For the first time we show that direct mast cell activation leads to different adult social interaction, and that prenatal inflammation leads to mast cell changes, which correspond to behavioral changes. Our results are consistent with epidemiology showing that early life inflammation increases risk for impaired social behavior, as seen in autism and other neuropsychiatric disorders.

**Figure 4:** Male Juvenile Play Following C48/80 Treatment



**Figure 4:** Juvenile play behaviors observed in male rats following PN1 C48/80 treatment



**Figure 5:** General schematic of experiment. We observe mast cells in the developing brain which peak in number around PN4. Activated mast cells release their content through degranulation. We hypothesize that alterations in the number of these cells will influence later life behaviors through developmental changes.

## Methods

### In vivo manipulations

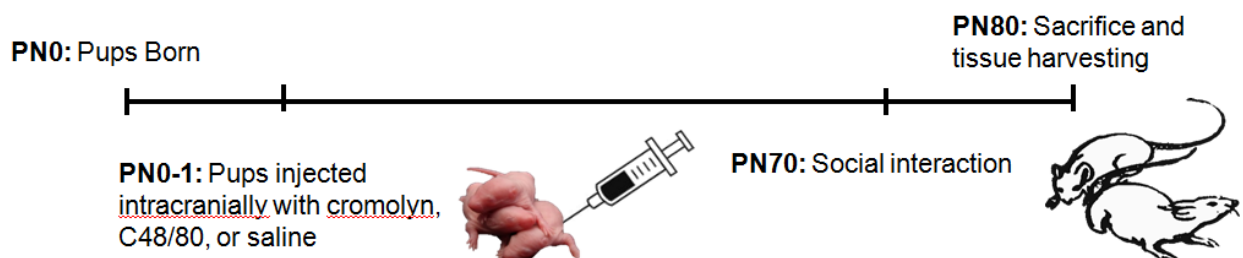
All breeding and experimental procedures were approved by The Ohio State University IACUC committee. Adult Sprague Dawley (Harlan) were mated in our facility, or timed pregnant animals were ordered to deliver approximately one week prior to parturition. Animals were maintained on a reverse 12:12h reverse light/dark cycle with lights off at 0800 hr, with *ad libitum* food and water in our vivarium. Pregnant rats delivered all pups naturally.

### Pharmacological manipulations using Cromolyn and 48/80 injections

Bilateral intracerebroventricular (i.c.v.) injections were performed under cryoanesthesia on the day of birth (PN0) and on PN1 (N = 49). A 23 gauge 1 µl Hamilton syringe attached to a stereotaxic manipulator was placed 1 mm caudal to Bregma and 1 mm lateral to the midline, lowered 3.0 mm into the brain, and then backed out 1 mm. A total of 1µl of drug or vehicle was infused over approximately 30 s, and the procedure repeated on the other hemisphere. For all procedures, the separation of pups from the dam was kept to a minimum, with i.c.v.-injected animals being separated for ~30 min. Each treatment group received two treatments of a mast cell stabilizer known as cromolyn sodium salt (cromolyn) (SigmaAldrich) to prevent mast cell degranulation (10 µg/µl; 20 µg total dose; n = 8 male, n = 7 female), a mast cell secretagogue known as compound 48/80 (SigmaAldrich) to induce degranulation (0.5 µg/µl; 1 µg dose; n = 10 male, n = 7 female), or vehicle (1 µl saline), n = 9 male, n = 8 female). Our previous studies showed the specified timing and dosing to be effective in mast cell regulation in the neonatal brain (Lenz and Galan, 2014). After neonatal treatment, animals used for behavioral testing were weaned at PN22 into sex-specific groups of two to three.

## Social Interaction

Social interaction test measures social exploration behaviors toward an unfamiliar conspecific animal. Adult social interaction paired a test animal (PN70, N=49, **Figure 6**) with an unfamiliar same-sex conspecific in a single 10 minute test. Four litters consisting of male vehicle (saline; n=9), cromolyn (mast cell stabilizer; n=8), and compound 48/80 (mast cell activator; n=10), female vehicle (n=8), cromolyn (n=7), and compound 48/80 (n=7) were used in the behavioral assessment. Social behavior in rats often depends on reciprocal behaviors of the other animals present, so a stimulus untreated conspecific was paired with the test animal. The animals were undisturbed and videotaped in a red-lit  $48 \times 38 \times 30$  cm (l  $\times$  w  $\times$  h) Plexiglas box during the dark phase between 0900 and 1500 hr. Behaviors recorded in the videos were scored blind to the treatment of the animal. Scored behaviors include: avoidance behaviors (when the test animal was approached by the stimulus animal and the test animal moved away), number of investigative behaviors (when test animal sniffed, initiated contact, or actively investigated the stimulus animal), amount of time investigating stimulus animal, and amount of time passively interacting with the stimulus animal (test animal was in direct physical contact with the stimulus animal but was directing no active investigation towards the stimulus animal).



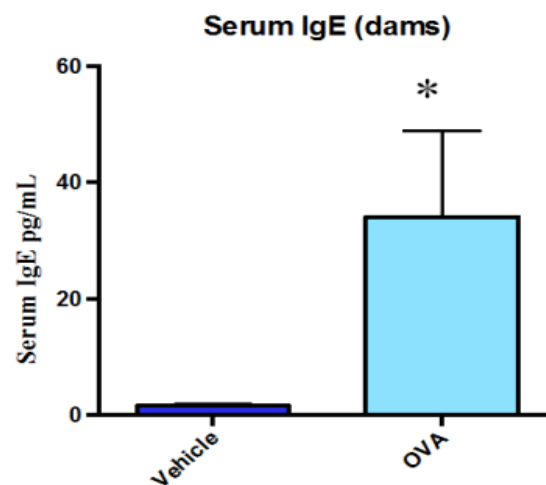
**Figure 6:** Timeline of neonatal pharmacological manipulations

## Pharmacological manipulations with Ovalbumin

Prior to pregnancy, adult females were sensitized with 1 mg ovalbumin injection (OVA grade V, SigmaAldrich) prepared at 4 mg/mL in pyrogen-free 0.9% saline and precipitated at a 1:1 ratio with  $\text{Al}(\text{OH})_3$  (Imject Alum, Thermo Scientific) according to manufacturer's instructions. After two weeks, a second 1 mg ovalbumin injection was given. A week later, females were paired with male for breeding. Detection of sperm signaled gestational day 0 (GD0). At GD15, pregnant rats were challenged intranasally with 1% ovalbumin (0.01g/ml) or saline solution (50 $\mu$ l), which was placed on each nare under light isoflurane anesthesia and inhaled upon regaining consciousness.

The females were observed for one hour following the challenge and showed no signs of sickness behavior, anaphylaxis, or other distress. We have previously demonstrated that this allergic challenge procedure produces a significant increase in serum IgE in the females 1 hr after the challenge (Lenz and Galan, 2014, **Figure 7**). Females were paired with one other

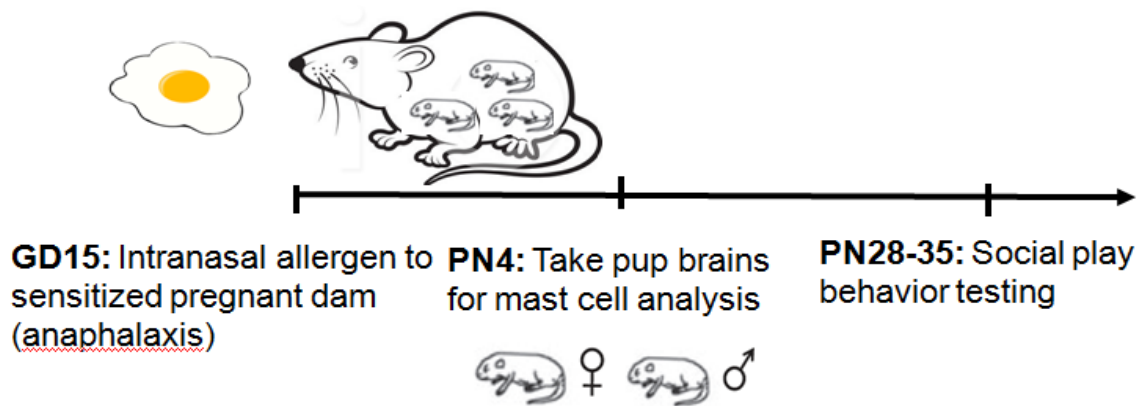
female rat until GD15 then were housed individually. After birth, animals used for behavioral testing were weaned at PN22 into sex-specific groups of three.



**Figure 7:** Serum levels in mother one hour after ovalbumin injection

## Social Play

Rough and tumble play behavior is a typical paradigm used in assessing juvenile social interaction (Auger and Olesen, 2009). Between PN28-38, ovalbumin-treated and related control animals were placed in mixed sex and treatment groups of six or seven animals, containing at least three males. Two ovalbumin treated litters (males n=10; females n=11) and one vehicle litter (males n=6; females n=5) were used. Rats were undisturbed and videotaped in the arena for 13 minutes to allow for a 3 minute habituation period and 10 minutes for behavioral scoring. Testing occurred daily for 5 days beginning ~1000-1300 hours during the dark phase (**Figure 8**). To differentiate between animals in the recording, each subject was given a unique identifying



**Figure 8:** Timeline of allergic challenge

symbol marked on their dorsal side with Sharpie marker. After marking, the rats were undisturbed for at least 40 minutes prior to testing. The entire group was placed in a bedded  $48 \times 38 \times 30$  cm ( $l \times w \times h$ ) Plexiglas arena under red light. After testing, rats were returned to their sex-specific home cages. Five behaviors - pouncing, chasing, wrestling, boxing, and pinning - were considered when scoring. Auger et al. defines the typical play behaviors we observed: “pouncing, the act of jumping on or placing both forepaws on the dorsal surface of another animal. A chase was counted whenever a rat made a sustained pursuit of another rat. Boxing



occurs when both rats stand on their hind paws and shove each other with their forepaws.

Wrestling occurs when two rats roll and tumble over each other. Pinning, or one rat holding another in a supine position, is often the result of boxing and wrestling bouts” (Auger and Olesen, 2009). Behavioral testing was videotaped and frequencies of play behaviors of each animal were quantified with experimenter blind to condition.

### **Tissue Collection and Analysis**

Brain tissue of pharmacological and ovalbumin treated adult and perinatal (PN4) rats was collected for histological analysis. For histology experiments, animals were injected intraperitoneally (i.p.) with FatalPlus (Vortech Pharmaceuticals), transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde, their brains removed and postfixed overnight in 4% paraformaldehyde, and cryoprotected with 30% sucrose until they sank. All brains were coronally sectioned on a cryostat into three alternate series at a thickness of 45  $\mu$ m. Sections were mounted on *Superfrost Plus* slides (FisherBrand). One series underwent histological staining for mast cells using toluidine blue (FisherChemicals). The stain highlights the metachromatic properties of the mast cell granules while lightly counterstaining the remaining neural tissue (Gokul and Shankar, 2012). Sections were washed in 60% EtOH for 2 min before they were stained in acidified 0.5% toluidine blue (pH=2.0). Excess stain was removed with a brief rinse in distilled water. Sections were then dehydrated with ascending alcohol (50% EtOH for 15 sec, 70% EtOH for 45 sec, 95% EtOH for 2 min, and 100% EtOH for 2 min.), defatted with xylenes (5 min,  $\times 2$ ), and coverslipped with Permount mounting medium (Fisher Chemicals).

## **Mast cell imaging and counts**

All sections were imaged using light microscopy with a 40× objective. Total mast cell counts were performed across the BNST, lateral septum, medial septum, amygdala, hippocampus, and habenula using computer based stereology software (StereoInvestigator, Microbrightfield) interfaced with a Zeiss AXIO Imager.M2 microscope and a Zeiss AxioCam MRm Digital Camera. All visible mast cells in the region of interest were counted manually. Distinctions between granulated and degranulated mast cells were also made. Granulated cells were characterized as darkly stained, concentrated granules, and a more circular shape. Degranulated cells showed lighter staining, more dispersed granules, and less defined structure (**Figure 2**).

## **Statistical Analysis**

For all datasets, individual numbers of social behaviors and numbers of mast cells were tabulated. Data were analyzed using two-way ANOVA tests and graphs were prepared using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Where significant effects were discovered using ANOVA, the Turkey's post-hoc comparison test was utilized to determine which groups were significantly different from one another. Alpha value was set at 0.05. All mast cells were totaled and normalized by multiplying the average number of cells per section by the animal with the largest number of sections to account for slight variations in number of sections per animal. The pharmacology experiments compared three factors when calculating statistics: vehicle, cromolyn, and compound 48/80 treatments. OVA experiments compared two factors, ovalbumin-treated and untreated animals.

## Results

### Adult mast cell numbers following early life pharmacological treatment

Two-way ANOVA revealed a significant sex difference ( $F_{1,8}=29.71$ ,  $p=0.0006$ ), significant overall effect between early life and adult mast cell numbers ( $F_{1,8}=1234$ ,  $p<0.0001$ ), and a significant interaction effect ( $F_{1,8}=27.27$ ,  $p=0.0009$ ). Turkey's post hoc analysis revealed significantly more mast cells in PN4

males as compared to PN4 females

( $p=0.0003$ ), PN4 compared to PN80

males ( $p<0.0001$ ), PN4 males

compared to PN80 females

( $p<0.0001$ ), PN4 females compared to

PN80 males ( $p<0.0001$ ), and PN4

females compared to PN80 females

( $p<0.0001$ ). Mast cell counts of the adult

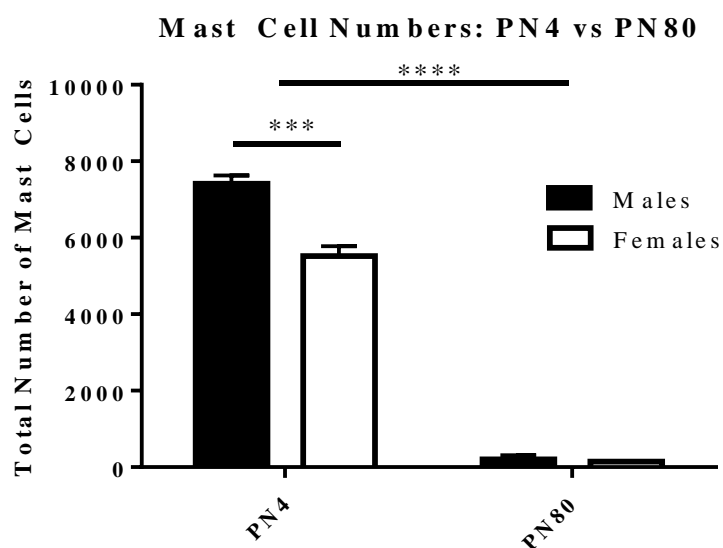
brain were shown to much lower in

adults than in the pups of all treatment

groups ( $p<0.0001$ , **Figure 9**). No significance was seen between PN80 males and PN 80 females.

### Social interaction following early life mast cell manipulations

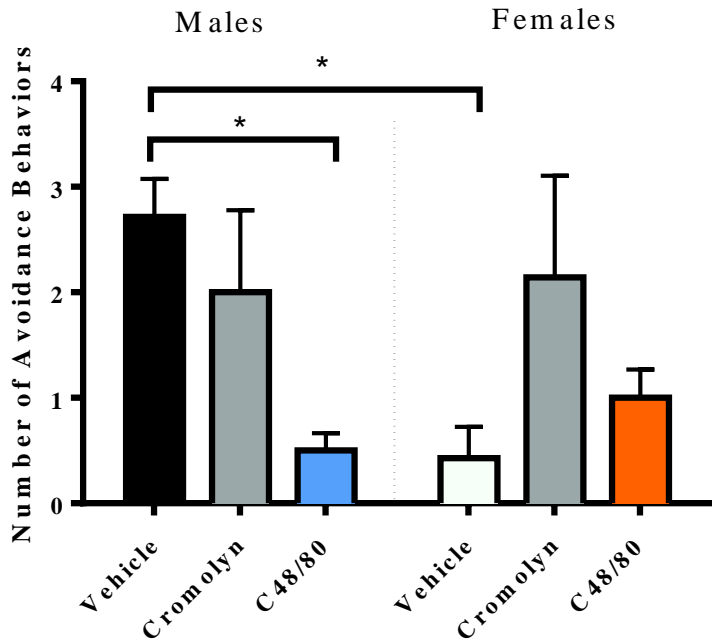
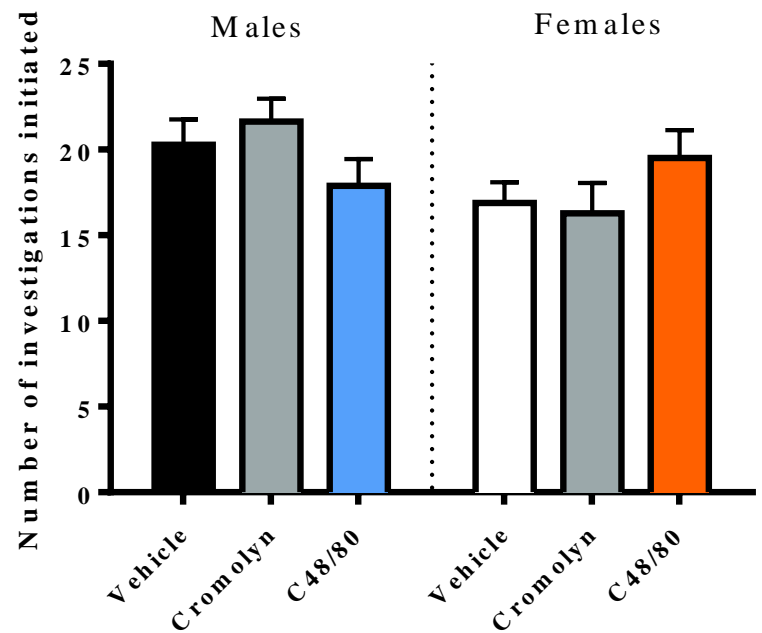
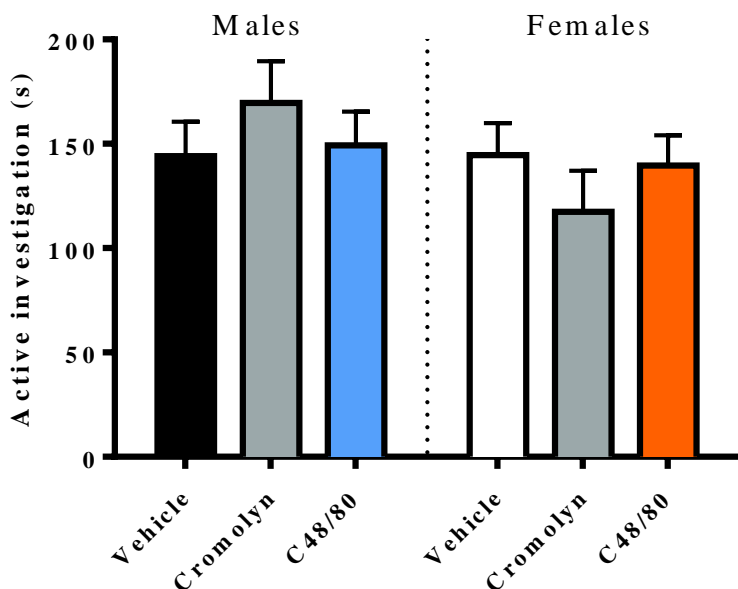
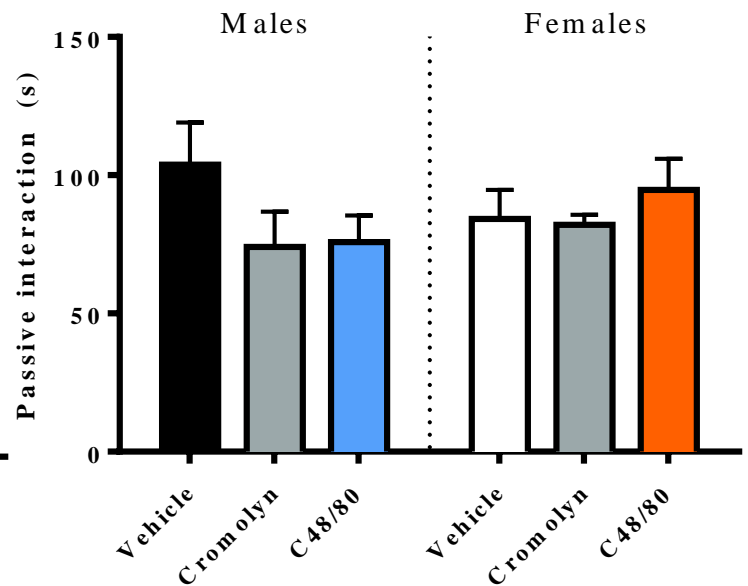
For social avoidance behavior, two way ANOVA revealed a significant main effect in pharmacological treatment in males ( $F_{2,41}=3.391$ ,  $p=0.0434$ ) but no sex difference ( $F_{2,41}=1.606$ ,  $p=0.2122$ ). There was also a significant interaction effect ( $F_{2,41}=3.965$ ,  $p=0.0269$ ). Post hoc analysis determined that social avoidance behaviors decreased in males after early life treatment with compound 48/80 compared (mast cell activator) to the control group ( $p=0.0422$ ; **Figure**



**Figure 9:** Number of mast cells observed in vehicle PN4 rats compared to number observed in vehicle adult brain.

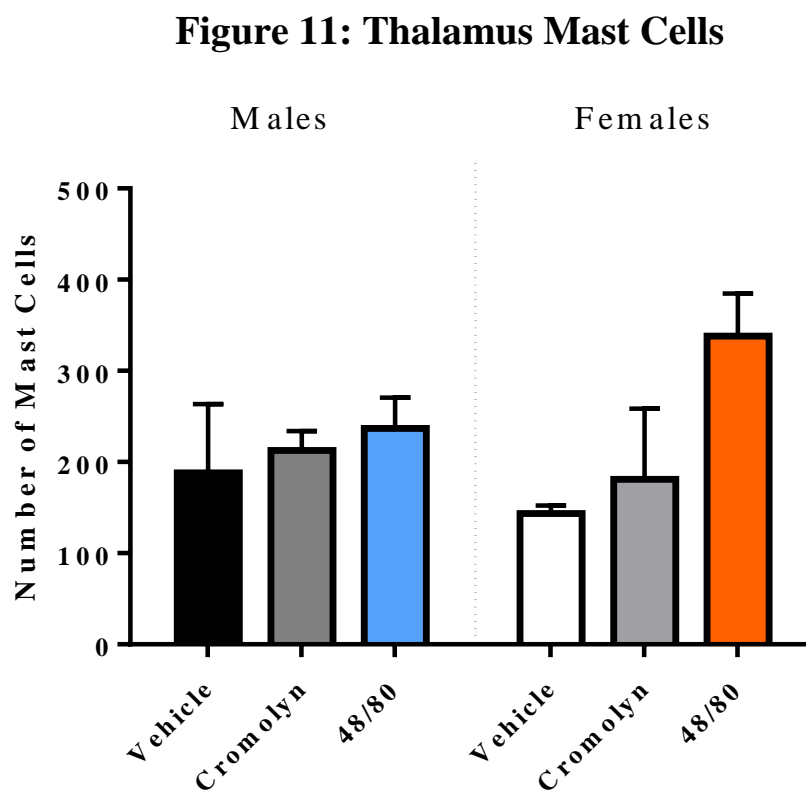
**10A).** No other treatment comparisons showed any significant differences (**Figures 10B-D**).

Upon visual analysis of these data, we excluded the cromolyn (mast cell stabilizer) treatment group which showed no significant difference from vehicles, to increase power. After doing so and re-performing two-way ANOVA, ANOVA revealed significant treatment difference ( $F_{1,28}=9.459$ ,  $p=0.0047$ ), sex difference ( $F_{1,28}=11.18$ ,  $p=0.0024$ ), and interaction effect ( $F_{1,28}=27.20$ ,  $p<0.0001$ ). Post hoc analysis once again determined that social avoidance behaviors decreased in males after early life treatment with compound 48/80 ( $p<0.0001$ ). Female vehicles showed significantly less avoidance behaviors compared to the male vehicles ( $p<0.0001$ ). Females treated with C48/80 showed fewer avoidance behavior than vehicle males ( $p=0.0007$ ). No other social interaction behaviors showed a significant difference. This includes number of active investigations, time actively investigating the stimulus animal, or time spent passively interacting with the stimulus animal.

**Figure 10A: Social Avoidance****Figure 10B: Active Investigation (bouts)****Figure 10C: Active Investigation (s)****Figure 10D: Passive Interaction (s)**

**Figure 10:** Social behaviors observed in the adult rats. All behavior was scored in one session paired with a same sex untreated conspecific. **10A:** Social avoidance was the only adult behavior to show significant differences. This behavior includes any effort made by the test subject to keep away from the stimulus conspecific. While the differences showed the expected sex difference, an increase, rather than decrease, in avoidance behavior was expected **10B:** Active investigation bouts include number of times the test rat approached the conspecific. **10C:** Time the subject performed investigative behaviors like sniffing or placing forepaws on conspecific. **10D:** Time the test rat was touching the stimulus animal but exhibited no investigative behaviors.

To assess any long term cellular changes, animals were sacrificed after the behavioral trials and the brain regions were compared. Mast cells were almost exclusively localized to the thalamus in adults and were quantified. Treatment with mast cell modulating drugs during the early perinatal period did not alter the number of mast cells in the adult thalamus ( $F_{2,10}=3.496$ ,  $p=0.0706$ ; **Figure 11**).

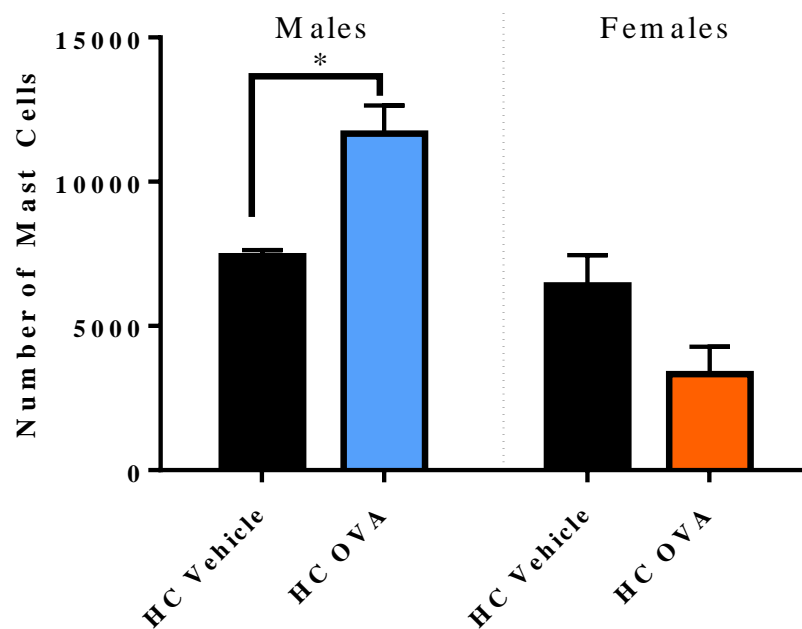


**Figure 11:** Number of mast cells in the adult thalamus. This was the only area that exhibited mast cells in the adult.

### Mast cell counts following prenatal allergic challenge

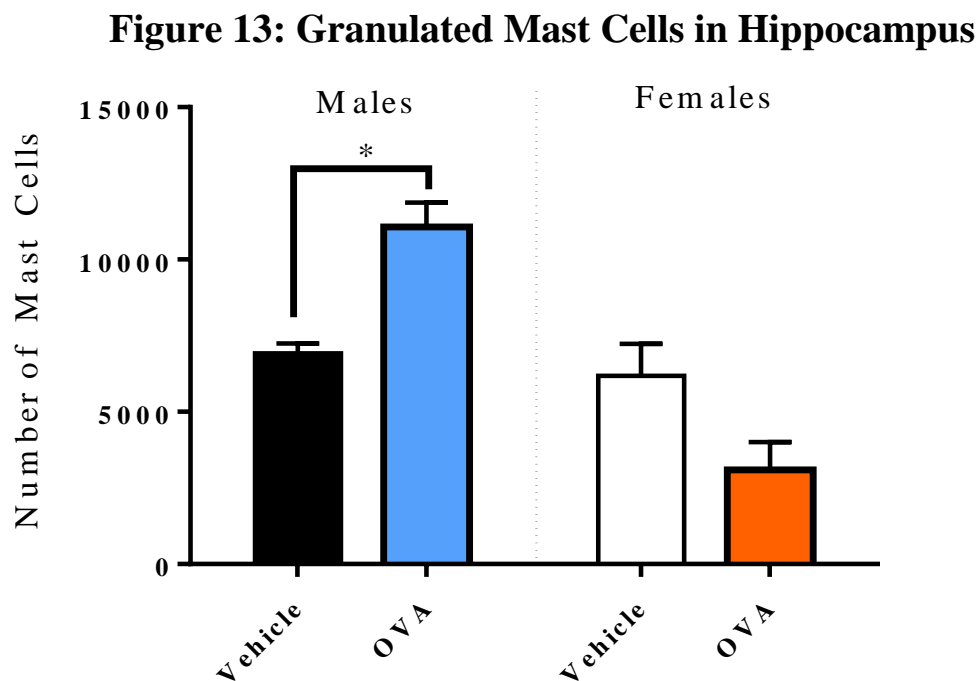
Prenatal allergic challenge significantly altered mast cell number in the postnatal hippocampal region (n= 3 per group). ANOVA revealed a significant total mast cell sex difference was also seen ( $F_{1,8}=29.32$ ,  $p=0.0006$ ), but no significant overall treatment difference was observed ( $F_{1,8}=0.4721$ , ns). A main interaction effect in number of total mast cells in the hippocampus was significant ( $F_{1,8}=18.05$ ,  $p=0.0028$ ). Post hoc analysis revealed significant differences in total hippocampal mast cells between: male vehicle (MV) and male OVA (MOVA) ( $p=0.0334$ ); MV and female OVA (FOVA) ( $p=0.0410$ ); MOVA and female vehicle (FV) ( $p=0.0110$ ); and MOVA and FOVA ( $p=0.0006$ ; **Figure 12**). No total mast cell differences were seen between other comparisons.

**Figure 12: Total Mast Cells in Hippocampus**



**Figure 12:** Total number of mast cells in the PN4 rat following gestational allergic challenge

The number of granulated mast cells in the hippocampus also showed overall granulated mast cell sex difference ( $F_{1,8}=28.41$ ,  $p=0.0007$ ), but no treatment difference was seen. ( $F_{1,8}=0.4267$ , ns). There was a significant interaction ( $F_{1,8}=19.68$ ,  $p=0.0022$ ). Post hoc analysis revealed significant differences in granulated hippocampal mast cells between MV and MOVA ( $p=0.0412$ ); MOVA and FV ( $p=0.0171$ ); and MOVA and FOVA ( $p=0.0006$ ; **Figure 13**). No total mast cell differences were seen between other comparisons.

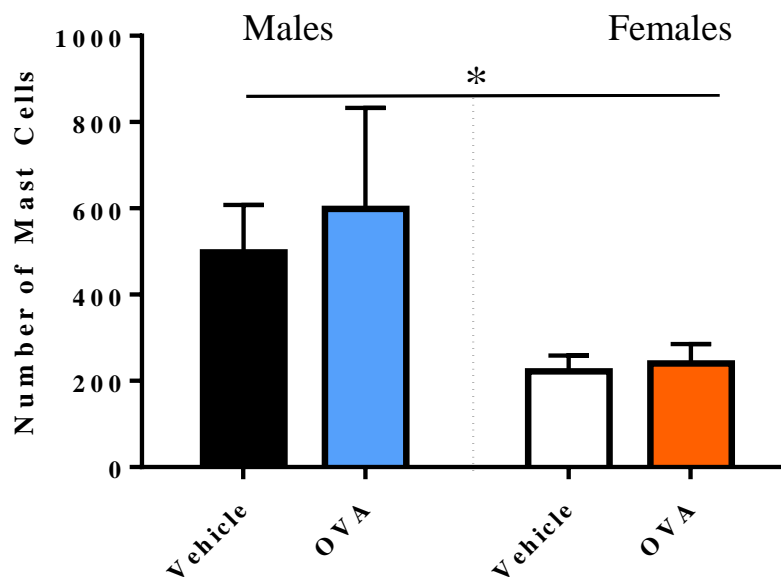


**Figure 13:** Number of granulated mast cells in the PN4 rat following gestational allergic challenge



In number of degranulated hippocampal mast cells, there was an overall sex difference ( $F_{1,8}=5.711$ ,  $p=0.0439$ ), but no overall significance between treatment groups ( $F_{1,8}=0.6677$  ns). No significant interaction effect was seen in degranulated hippocampal mast cells ( $F_{1,8}=0.0967$ , ns; **Figure 14**). Post hoc analysis revealed no other significant comparisons.

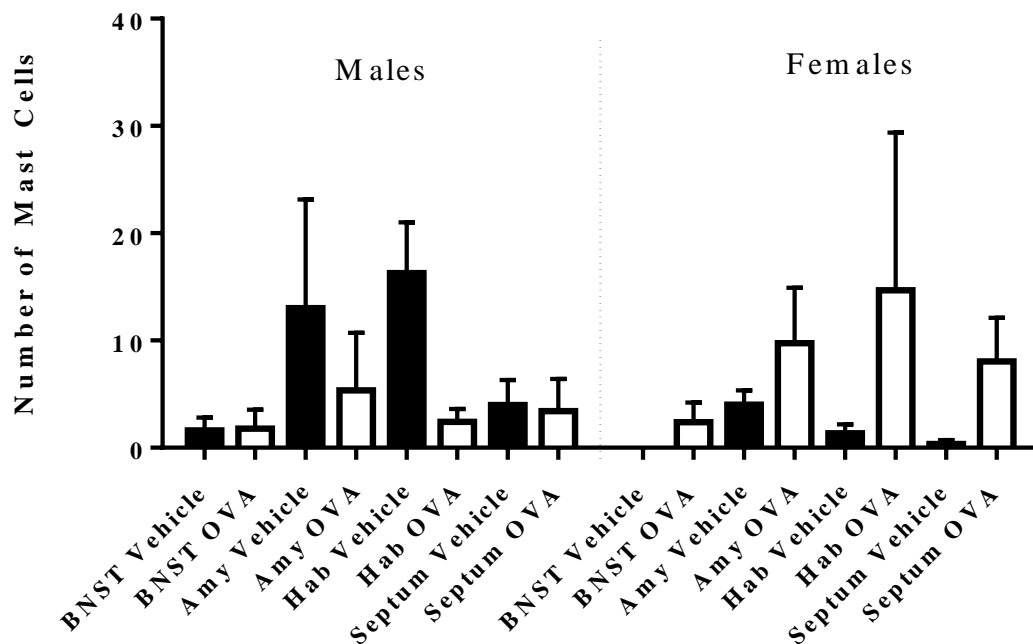
**Figure 14: Degranulated Mast Cells in Hippocampus**



**Figure 14:** Number of degranulated mast cells in the PN4 rat following gestational allergic challenge

Other areas in the brain thought to be involved with behavior also showed no significant differences in total number of mast cells by sex, treatment, or interaction: amygdala ( $F_{1,8}=1.114$ , ns, **Figure 15**), BNST ( $F_{1,8}=0.6040$ , ns), habenula ( $F_{1,8}=3.080$ , ns), and lateral and medial

**Figure 15: Total Mast Cells in Other Regions**



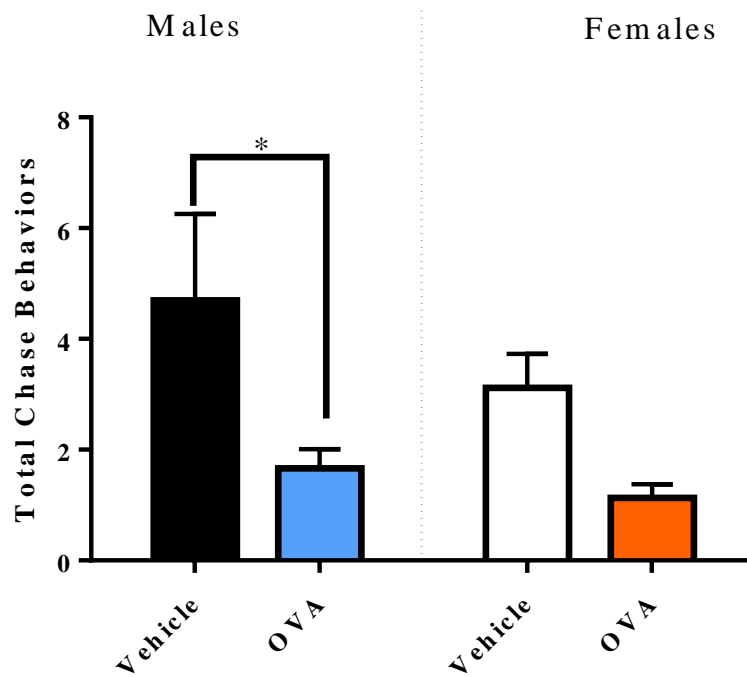
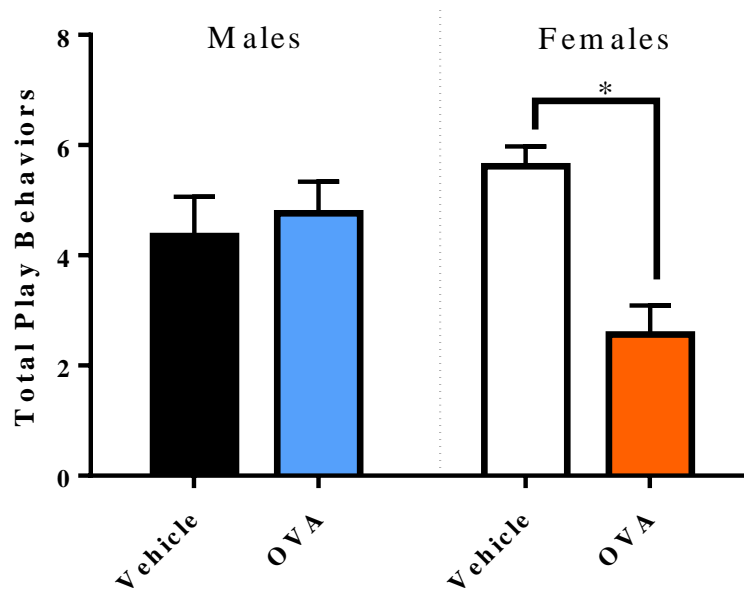
**Figure 15:** Number of total mast cells observed in the PN4 rat following gestational allergic challenge in other areas previously shown to play a role in social behavior. Bed nucleus of stri terminalis (BNST); amygdala (amy); habenula (hab); lateral septum (septum)

septum ( $F_{1,8}=2.208$ , ns). These other areas showed no significant sex differences in total number of mast cells: amygdala ( $F_{1,8}=0.1303$ , ns), BNST ( $F_{1,8}=0.1236$ , ns), habenula ( $F_{1,8}=0.0287$ , ns), and lateral and medial septum ( $F_{1,8}=0.0338$ , ns). They also showed no significant treatment differences in total number of mast cells: amygdala ( $F_{1,8}=0.0230$ , ns), BNST ( $F_{1,8}=0.8146$ , ns), habenula ( $F_{1,8}=0.0011$ , ns), and lateral and medial septum ( $F_{1,8}=1.641$ , ns).

## **Prenatal allergic challenge behavior**

In total, the behavior of OVA males (MOVA) and eleven OVA females (FOVA) derived from two litters was compared to the behaviors of six male vehicle (MV) and five female (FV) vehicle pups from one litter. The data were averaged across groups and normalized for minor differences in the number of trials. ANOVA showed that prenatal treatment with ovalbumin had no significant overall sex differences ( $F_{1,28}=1.382$ , ns), but revealed significant treatment effects ( $F_{1,28}=21.87$ ,  $p<0.0001$ ). No interaction ( $F_{1,28}=2.407$ , ns) were seen. Post hoc analysis revealed significance in MV and female OVA (FOVA) ( $p=0.0010$ ) and between FV and FOVA ( $p=0.0010$ ). No other comparisons were significant.

To determine if specific play behaviors were affected by the allergic challenge, we looked at chasing behavior separately from the other rough and tumble behaviors (boxing, pinning, pouncing, and wrestling). ANOVA revealed no significance between sex ( $F_{1,28}=2.286$ , ns) but did show an overall effect between treatment groups in chase behavior ( $F_{1,28}=12.89$ ,  $p=0.0012$ ). There was no overall interaction effect ( $F_{1,28}=0.5577$ , ns). Post hoc analysis showed significance decreases in MOVA compared to MV ( $p=0.0197$ ) and in FOVA compared to MV ( $p=0.0043$ ) in chasing behavior. No differences were seen between other comparisons. ANOVA revealed no overall sex difference between groups in rough and tumble play behaviors ( $F_{1,28}=0.5586$ , ns), but did show an overall treatment difference ( $F_{1,28}=4.458$ ,  $p=0.0438$ ). There was also an interaction effect ( $F_{1,28}=7.675$ ,  $p=0.0098$ ). Post hoc analysis showed significance decreases in female vehicle versus female OVA ( $p=0.0101$ ) and in male OVA versus female OVA ( $p=0.0268$ ), while no differences were observed between other comparisons. **(Figure 16A and B).**

**Figure 16A: Juvenile Social Chase Behaviors****Figure 16B: Juvenile Social Play: Rough and Tumble**

**Figure 16:** Number of chase behaviors (A) and rough and tumble play behaviors (B) observed in the juvenile rats (PN28-35) following allergic challenge

## **Discussion**

In this study, we focused on an area not previously explored: how changes in mast cells during development influence later life behaviors in the juvenile or adult rat. In the first pharmacological manipulations, we found that activation of mast cells decreased social avoidance in adult males while females went unaffected. These data correspond to the previous study in our lab where social play behavior of juvenile males, but not in females, was decreased. However, these results were partially unexpected because the autistic phenotype would be expected to avoid social contact. We followed this study by looking how prenatal inflammation would affect the same behaviors. In the juvenile rats, we saw our expected results that ovalbumin challenged rats displayed a decrease in social behaviors, and males saw a decrease in social chase behaviors. These differences corresponded, as expected, to an increase of mast cells. However, it was unexpected to see a decrease in rough and tumble play in the females while no difference was seen in the males.

## **Pharmacological treatment**

Our first experiment to test the involvement of mast cells in programming later life behavior showed a significant decrease in social avoidance in male rats when mast cells were activated by C48/80 at PN0-1. Additionally, male and female vehicles displayed a sex difference. Previous work in our lab, using the same paradigms used to assess juvenile behavior in allergic challenge animals, found that males showed social play was reduced by 40%, but no differences in females were observed when treated with the same mast cell degranulating agent (Lenz and Galan, unpublished). The sex difference in behavioral change carried over in this study's observation that rats with early life mast cell activation exhibited significantly less social interaction as adult males. These results show that by directly stimulating these cells at an early

developmental time point mast cells play some role in the developing brain that can preprogram lifelong behavioral changes.

Several studies have demonstrated that males are more vulnerable to changes during development that contribute to neuropsychiatric disorders (Bale, 2011; Taylor et al., 2012). One explanation suggests the existence of currently unknown modulating factors present during gestation that either serve as a protective factors in females or a detrimental factors in males or some combination of the two (Marshall, 2004; Pietrzak et al., 2011). With mast cells capable of producing such a wide variety of regulatory compounds, their products might contribute to such changes. Since male behaviors were affected more than females' with drug-induced mast cell activation, this points to the conclusion that mast cell activation plays a role in development of areas associated with social behavior. The fact that males also have significantly more mast cells without any treatments and that dysregulation of this cell type induces sex biased behavioral changes supports the hypothesis that mast cells might be an underlying factor for the sex bias observed in neuropsychiatric disorders. We do not yet know whether the sex difference is programmed directly by male-typical hormones during development, by sex chromosomes, or whether mast cells are recruited differentially into the brain via other local cells, such as microglia, neurons, astrocytes, or endothelial cells of the blood brain barrier.

However mast cell stabilizer cromolyn showed no significant effect in either sex. One explanation might be the effectiveness of cromolyn, the dose used, or the dosing schedule. Cromolyn has been shown to have varying degrees of effectiveness in blocking degranulation in peripheral mast cells (Barrett and Metcalfe, 1985), so completely blocking the mast cells might not have been achieved allowing for any necessary developmental effects to be compensated by other mast cells. Also administering the drug i.c.v. could have induced local inflammation and

non-specific activation of mast cells or other immune cells to counteract the effectiveness of pharmacological mast cell inhibition. Future studies could address this by giving cromolyn systemically or increasing the dose given.

Furthermore, the adult reduction of social avoidance after mast cell activation is not necessarily indicative of autistic-like behavior, since behavior typically includes aversion to touch in humans (American Psychiatric Association, 2013; Silverman, 2010). This could indicate that the mechanism underlying this social behavior might be more influential at an earlier time point during development. On the other hand, since the disease falls on a spectrum, few if any individuals and no current model exhibit every characteristic of autism (American Psychiatric Association; Silverman, 2010).

Aside from social interaction, no other behaviors were affected. Confounds of both drugs might be possible due to timing of administration. Since the drugs would have been exerting their effects on PN0-1, brain development would more complete than when rats are challenged prenatally at GD15, meaning if mast cells had effects on hippocampal development, they would be less effective following drug administration since any neural networks would be much more strongly formed. Moreover, each drug does not necessarily resemble any naturally occurring environment. Even though compound 48/80 and cromolyn have been shown to act on the mast cell's FcεRI, the same receptor activated by IgE (Shapiro and P König, 1985), true inflammation would likely have impacted the surrounding microglia and elicited other inflammatory factors to be spread throughout the developing parenchyma. Since the compounds synthesized by the mast cells are often determined by the environment (Galli et al., 2005), stimulating degranulation without other inflammatory markers does not model the mast cell role in development. Future experiments will address this by co-administering a systemic immune challenge with LPS and

concurrently inhibiting mast cells with cromolyn to tease out the specific effects of mast cells on the development of social behavior.

### **Allergic challenge**

The allergic challenge sought to expand on the results of our pharmacology experiment by replicating a more biologically relevant factor. Inflammation that has been shown as a risk factor for autism is usually marked by an elevation in a wide range of cytokines and other modulating factors. By sensitizing and challenging the mother during gestation with ovalbumin, we mimicked the typical allergy- related immune response that might be seen during pregnancy in humans.

Prenatal allergic challenge led to decreased social behavior in males and females. When challenged with OVA during gestation, male juveniles chased significantly less while their rough and tumble behavior did not change. The decline in social chase behavior in juvenile males correlated to the increase of mast cells in the hippocampus. Both total and granulated mast cells showed a significant increase in the males while remained more stable in the females. Although the number of degranulated mast cells did not produce statistically significant results, adding more subjects to the data would likely show significant differences. With both the pharmacological degranulator and allergic challenge, male behavior significantly changed, suggesting a link between increased number of mast cells mast, and therefore their mediators, and later life social behavior. The females displayed a seemingly opposite trend, since OVA female chase behaviors did not show significance while their rough and tumble behavior did show significant decline. These results were unexpected. Further studies will look to add subjects in both the social play and mast cell counts.



Decreases in juvenile male chasing behavior is reminiscent of the decline in social interaction in autism. However, no decrease was seen in rough and tumble behaviors. Females displayed a decline in rough and tumble play while no differences in chase behavior were seen. Even though these results were unexpected, they show that both behaviors can be influenced by inflammation, but the sex bias may influence how the effects of that inflammation presents itself in the more developed brain.

Our lab is also about to assess the differences in behavior of OVA treated adults. While treatment with the mast cell stimulator C48/80 exhibited unexpected results of decreasing social avoidance, our lab is in the process of assessing adult social interaction of adults. If the behavioral results correlate to the findings observed in C48/80 treatment, mast cells would be shown to have an effect on this behavior throughout life. If the behaviors exhibit an opposite trend, we could infer that allergy treated animals had a longer recovery time or some compensation mechanism developed following treatment. To separate the two behaviors, future studies would look at one on one interaction at the juvenile stage to see if timing of drug delivery has an influence on this behavior.

While the other areas previously implicated in behavior modulation, such as the BNST, habenula, amygdala, and septum, share connections with the hippocampus, the very low number of mast cells located in those areas suggest that any influence neuroinflammatory processes play in their development or role in neuropsychiatric disorders is not directly regulated by mast cells in these regions themselves. However, the projections to them from the hippocampus could influence their activation. Future studies will use immediate early gene analysis using c-Fos to see which brain regions show acute activation following mast cell stimulation or allergic challenge.

With the allergic challenge, other immune cells present in the brain will also be activated, which would add neuroactive compounds into the developing brain from sources other than mast cells. While this might seem contradictory to the confound that pharmacological treatment does not mimic proper environmental conditions, the precise compounds released by the mast cells in the CNS under untreated or inflammatory conditions has not been discovered. Further studies will seek to qualitatively and quantitatively examine what contents the mast cells release at various time point and manipulate those factors directly.

### **Conclusion and future directions**

Activation in male mast cells through both pharmacological and inflammatory challenges suggests that the mast cells may have a greater impact on later life male behavior than the females. One recent discovery shows that mast cells have estrogen receptor alpha, which could explain the sex difference exhibited by mast cells. That being said, our results showing that female behavior changes after gestational inflammation but without mast cells changes leads to the conclusion that is highly unlikely any one factor is sufficient to induce the developmental and behavioral changes observed in autism. It is more probable that some complex interaction between genetics and the environment work together to disrupt the developmental process at some point to produce the behavioral phenotype observed in the disease, but our results demonstrate that mast cells in the hippocampus are one possible factor that is disrupted prior to ontogeny.

Although the exact cause of autism, schizophrenia, and other developmental disorders are not fully characterized, dysregulation of the immune system in the developing brain are risk factors for the onset of these diseases. Throughout life, myeloid derived immune cells reside in the brain and release many neuroactive compounds that also play a role in development (Cote et

al., 2007, Galli et al., 2005). During development, the primary mast cell population resides in the same region as many microglia, supporting the idea that microglia and mast cells interact during development. Overall, we show that mast cell modulation during early life does disrupt the typical behaviors, and many of those behaviors resemble the autistic phenotype. Furthermore, inflammation influences the mast cell population and behavior, particularly in males.

For inflammatory conditions, future studies will look at the effects of LPS and cromolyn combined to find if the main source of the inflammation is due to mast cell activation. If our methods can be refined, mast cells seem like a sensible target for research on developmental disorders. One such way may even rely on cromolyn sodium, since it is already an FDA approved drug though only for treating asthma. If future tests continue to point towards mast cell dysregulation as a source of behavioral changes, this drug might serve as a larger therapeutic in combating the onset autism in children at risk.

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